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Vaccine from the C-Terminal Fragment of Plasmodium
Falciparum 3D7 Merozoite Surface Protein-1

PRINCIPAL INVESTIGATOR: Evelina Angov, Ph.D.

CONTRACTING ORGANIZATION: Evelina Angov, Ph.D.
Bethesda, Maryland 20184

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FOREWORD

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TABLE OF CONTENTS

SF 298 Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5
Conclusions	17
References	19
Bibliography	21
Figure 1	22
Figure 2	23

INTRODUCTION

Over 300 million cases of malaria are reported each year worldwide, resulting in over 3 million deaths mostly among young children. The leading cause of clinical malaria is through infection with the parasite *Plasmodium falciparum*. Malaria caused by *P. falciparum* can lead to serious clinical illness and if untreated to death. Development of effective controls against malaria, either through the mosquito vectors or the parasite has lead to widespread pesticide and drug resistance, respectively. As these approaches have been unsuccessful, efforts have focused on malaria vaccine development.

Infection of *P. falciparum* through the mosquito vector initiates the complex life cycle within the human host. The parasite passes through progressive developmental stages that arise following invasion of the liver. Parasitic release from liver cells initiates the erythrocytic stage of the parasite life cycle. During the erythrocytic stage, red blood cells are invaded. Maturation and expansion of parasites, the merozoites during this stage, occurs within the red blood cells sequestered away from the host immune system. Vaccine candidates have been identified from each of the parasite's developmental stages. A leading erythrocytic stage candidate is the major merozoite surface protein, MSP1, [Diggs, et al., 1993]. Vaccines derived

from malaria erythrocytic-stage antigens like MSP1 are of special interest because erythrocytic stages are the only confirmed targets of natural immunity among individuals from malaria endemic regions. Therefore, development of an efficacious erythrocytic stage vaccine from MSP1 or the C-terminal fragment (MSP1₄₂) has the potential to protect non-immune individuals. Since malaria-naïve individuals do not possess partial immunity developed through life-long exposures, immunization with MSP1₄₂ could induce the development of antibodies that are qualitatively comparable to those developed from natural malaria exposures. The mechanism of protection induced by an erythrocytic stage malaria vaccine would be mediated through the development of specific protective antibodies to proteins on the surface of parasites. Antibodies raised to parasite surface proteins would lead to an inability of the erythrocytic stage parasites (merozoites) to re-invade new erythrocytes. The suggested mode of action of these antibodies is to bind to the surface of the merozoites and block their ability to associate with, and invade erythrocytes, or to interfere with biochemical events associated with invasion. Blocking invasion would reduce the amplification of parasites in the bloodstream and thus reduce the overall parasitic load and severity of disease. Therefore, the development of specific antibodies to erythrocytic stage antigens like MSP1 could reduce

the likelihood of serious illness and disease in malaria-naïve individuals.

MSP1's function is not well understood [Holder and Blackman, 1994; Holder, et al., 1988; Miller, et al., 1993]. MSP1 is synthesized as a large 195-kDa precursor protein. It is anchored to the surface of the merozoite through a C-terminally attached N-glycosylphosphatidylinositol (GPI) anchor [Haldar, et al., 1985]. Following release from red blood cells, proteolytic processing of full length MSP1 yields products with nominal molecular masses of 83, 28-30, 38-45 and 42 kDa. These processed polypeptides are recognized by MSP1 specific antibodies [Holder and Freeman, 1984; Lyon, et al., 1986; Holder, et al., 1987]. The MSP1 derived polypeptides remain noncovalently associated with each other at the surface of merozoites and are directly attached to the merozoite surface through the MSP1 42kDa C-terminal fragment [McBride and Heidrich, 1987; Lyon, et al., 1987]. Either just before or during erythrocyte invasion, C-terminal MSP1₄₂ is processed again to a 33 kDa fragment and a 19 kDa C-terminal fragment (MSP1₁₉) [Blackman, et al., 1991]. The non-covalently associated polypeptide complex is shed from the merozoite surface leaving only the 19 kDa fragment anchored through GPI. Following erythrocyte invasion, only MSP1₁₉ is present on ring forms in the newly invaded erythrocyte [Blackman, et al., 1990].

The C-terminal MSP1₁₉ has a rather complex structure. Twelve cysteine residues occur within a span of 100 amino acid. Sequence alignment data suggests that two tandem domains form having homology at the level of cysteines with epidermal growth factor (EGF) [Blackman, et al., 1991]. Each EGF-like domain contains six cysteine residues that could form three disulfide bridges per domain however, neither the number of disulfide bridges nor the linkage pattern of the disulfides has been directly confirmed on native MSP1.

Development of specific antibody responses to native MSP1 molecules requires that important conformational epitopes be present on the surface of these molecules. Several lines of evidence support the use of MSP1, and the C-terminal fragments, MSP1₄₂ and MSP1₁₉, as components of erythrocytic stage malaria vaccines. First, MSP1₁₉-specific monoclonal antibodies inhibit *P. falciparum* growth *in vitro*, [Blackman, et al., 1990], or passively protect mice against infection with *P. yoelii*, [Majarian, et al., 1984; Ling, et al., 1994]. Second, immunization of monkeys with native MSP1, [Siddiqui, et al., 1987], baculovirus-expressed recombinant MSP1₄₂ [Chang et al., 1996], or *S. cerevisiae*-secreted recombinant MSP1₁₉ (EVE-MSP1₁₉) from *P. falciparum* [Kumar, et al., 1995], can protect against a homologous challenge. Similarly, *E. coli*-expressed recombinant MSP1₁₉ from *P. yoelii*, [Holder, et al., 1994; Burns, et al.,

1989] protects against a homologous murine challenge. Finally, anti-sera raised against recombinant MSP1₄₂ [Chang, et al., 1992], or MSP1₁₉ [Lyon and Haynes, unpublished] inhibit *P. falciparum* growth *in vitro*. The MSP1₁₉-specific monoclonal antibodies that either protect against infection *in vivo* [Burns, et al., 1989], or inhibit parasite growth *in vitro* [Blackman, et al., 1990], are specific for discontinuous epitopes since they do not react with disulfide-reduced MSP1₁₉ [McBride and Heidrich, 1987; Farley and Long, 1995]. Some monoclonal antibodies directed to MSP1₁₉ have been shown to inhibit parasite growth by blocking the proteolytic processing from 42kDa to 33kDa and 19kDa. A second class of monoclonal antibodies inhibits the ability of blocking antibodies and allows proteolytic processing to occur [Patino, et al.; 1997]. Therefore, the ability of antibodies to neutralize parasite development is dependent on the type of specificity developed to epitopes on the C-terminal MSP1₁₉. The development of antibodies with opposing specificity's may have arisen as a mechanism to avoid host immune responses. Thus, a recombinant vaccine product from this region of MSP1 may not only require correct disulfide-dependent conformation but selective development of specific antibodies to elicit a protective antibody response.

Successful expression of heterologous proteins in *E. coli* can lead to high levels of recombinant proteins. Bacterial

expression has the advantage of being relatively inexpensive, and is readily scaleable. Proteins expressed in bacteria are not post-translationally modified and heterologous proteins that require these modifications may have altered activities.

Eukaryotic expression systems, such as yeast, baculovirus, or mammalian cells, post-translationally modify, however, the modifications may not be correct and protein yields can be poor.

Induction of immune responses using heterologously expressed recombinant proteins requires that protein conformation and structure is correct. Heterologous expression of some recombinant MSP1 molecules (MSP1₄₂ and MSP1₁₉) from eukaryotic expression systems, i.e. baculovirus and yeast, have lead to recombinant proteins that are either correctly folded and expressed poorly or are mis-folded and expressed well, respectively. To avoid some of these issues, well-described bacterial expression systems (pET Expression System from Novagen) were used to express the C-terminal MSP1₄₂ fragment.

The objective of this work was to develop erythrocytic stage-specific malaria vaccine candidates that could elicit protective antibodies in volunteers in Phase I trials. Since MSP1 is a rather large protein, bacterial vaccine strategies have focused on proteolytic fragments. *In vitro* studies have shown that some protective epitopes on MSP1 may be contained within the C-terminal MSP1 cleavage product, MSP1₄₂. The C-

terminal MSP1₁₉ folds into a rather complex tertiary structure. Therefore, presentation of relevant epitopes on the C-terminal MSP1₁₉ is dependent on correct folding and conformation of MSP1₄₂. Recombinant plasmids were transformed into *E. coli* expression host, BL21(DE3) and recombinant proteins were expressed. Purification of recombinant MSP1₄₂ was through metal affinity chromatography using a six-histidine (His₆) amino acid sequence tag cloned at the N-terminus of the MSP1₄₂. Additional chromatography included anion exchange chromatography to remove endotoxin and nucleic acids and finally a cation exchanger, carboxymethyl resin, to concentrate the protein. Purified MSP1₄₂ will be formulated with an appropriate adjuvant and used in immunization studies of non-human primates and a human Phase I clinical trial to investigate the development of humoral immune responses.

The production of recombinant molecules using DNA technology and large-scale fermentation processes has enabled the development of proteins in quantities otherwise impossible. Although previously it was difficult to use bacteria to express some complex eukaryotic proteins, recent advances in the design of bacterial strains and the development of tightly regulatable expression vectors have lead to their use in expression of some complex eukaryotic proteins.

The construction of an MSP1₄₂ (3D7) expression vector to express and purify recombinant MSP1₄₂ proceeded through a series of vector constructions that ultimately led to the final clone, designated pET42AT(NK2), His₆-MSP1₄₂(3D7) (Figure 1). The final construct, so developed, was designed to meet the specifications required by the FDA for a product developed for human use from *E. coli*. The final His₆-MSP1₄₂ product contains a short N-terminal fusion on MSP1₄₂ that encodes six histidine residues and 11 linker amino acids. The plasmid also contains the gene for tetracycline selection. The detailed description of the construction of the DNA vector expressing a *P. falciparum* 3D7 MSP1₄₂ has been described elsewhere (Annual Report 1997).

Early stages of the process required optimization of fermentation and induction of expression of recombinant MSP1₄₂. A three hundred-liter scale GMP fermentation performed in June 1998 at Forest Glen Annex, Division of Biologics Research, provided adequate amounts of wet cell paste for purification process development. Optimal fermentation processes applied to large-scale fermentation can be empirically derived from small-scale culture conditions. Composition of the culture media, culture temperature, cell density at which induction of expression occurred, could have significant effects on the levels of protein produced and the total cell mass. Many heterologous proteins expressed in *E. coli* are either expressed

at very low levels, or insoluble in inclusion bodies. Expression of MSP1₄₂ was at good levels and the protein remained soluble when induction occurred at 25°C.

Affinity chromatography using Ni⁺² chelating resins provides a simple, highly specific elution of desired target proteins. Six consecutive histidine residues are expressed as a short N-terminal fusion on the target protein. The application of cleared soluble supernatant fractions onto the Ni⁺² agarose resin, allowed separation of other *E. coli* proteins from the recombinant His₆-MSP1₄₂. The His₆-MSP1₄₂ protein bound tightly through the imidazole ring in the histidine residues to the nickel ions immobilized by the NTA (nitrilotriacetic acid) (Qiagen) groups on the resin. Increasing concentrations of free imidazole compete with the His₆-MSP1₄₂ protein for binding to the matrix and result in elution of the tagged protein. Following this first chromatographic step, the MSP1₄₂ protein was present at greater than 60% purity. Additional chromatography was required to purify the protein to near homogeneity. Following extensive purification process development, MSP1₄₂ was purified to near homogeneity using two additional chromatographic steps; an anion exchanger, SuperQ 650M (TosoHaas), followed by a cation exchanger, CM650M (Tosohaas). The anion exchanger functioned to reduce the levels of endotoxin and nucleic acids present in the protein. The cation exchange

step removed a major *E.coli* contaminant and concentrated the protein. This 3-step chromatography yielded greater than 93% purity in MSP1₄₂.

During the course of the contract year, 1998, the MSP1₄₂ was scheduled for purification at the GMP scale on two separate occasions. In November, a 1.5kg wet cell paste was used to purify the MSP1₄₂ under the above-described conditions. The process was performed under nonGMP conditions, however, the process was performed at near GMP conditions, and with the exception, that formal documentation was not provided. This purification was performed to test the reproducibility of the purification process and to determine whether the process was scaleable. The SDS-PAGE and western blotting data from the November nonGMP purification showed that the protein was obtained at high levels of purity, however, the protein contained unacceptable levels of endotoxin (30EU/dose acceptable, November nonGMP, 60EU/dose). Endotoxin levels were determined both by *in vitro* gel clot LAL (*Limulus ameobocyte* lysate) and rabbit pyrogenicity tests. High levels of endotoxin suggest that the purification process was not sufficiently rigorous. Under optimal conditions, endotoxin is removed from aqueous solutions and some protein samples using anion exchange chromatography due to electrostatic attractions. In this case, although the endotoxin levels were not acceptable for animal

studies or human use, it was felt that with some modifications to the BPR (Batch Production Record), the endotoxin levels could be reduced to acceptable levels. Therefore, a second GMP purification scheduled for December would proceed. The changes made to the process were to increase the number of column volumes used to wash the Ni^{+2} column following the sample application. The data show that following this production, the level of protein purity was greater than 95% as measured by scanning densitometry (Figure 2 Coomassie Blue stained gel, western blots probed with mAb7F1 and mAb12.10). Immunoreactivity against MSP1₄₂ is with the major Coomassie blue stainable band and aggregated forms of MSP1₄₂. Gel clot LAL and rabbit pyrogenicity assays showed an even greater level of endotoxin, (600EU/dose). Since the endotoxin levels did not meet the release specifications for the final product, animal studies and the submission of the IND to the FDA was postponed pending re-evaluation of the purification process.

Work was initiated to re-develop the purification process of MSP1₄₂ with special attention to optimization of endotoxin removal at each chromatographic step. Review of the nonGMP and GMP purification BPR's and evaluation of an earlier to-scale purification performed in September 1998, showed that specific changes in buffer pH and detergent could lower endotoxin levels. Chromatography at pH 6.2 on Ni^{+2} metal chelate should result in

lower affinity of endotoxin for MSP1₄₂ and the column. At this pH the MSP1₄₂ closer to it's isoelectric point and has a reduced charge. The Ni⁺² column would be weakly positively charged and endotoxin would easily pass through. Detergents, Tween 80, present in the buffers prior to cell lysis by microfluidization would reduce the interaction of endotoxin with proteins. In addition, Tween 80 was included in the dilution buffer and equilibration buffer for the anion exchanger, SuperQ. Detergents present during sample application may dissociate protein/endotoxin complexes. A last change was to lower the sodium chloride concentration during sample application on the SuperQ resin. This change increases the affinity of endotoxin binding to the SuperQ. These changes were introduced to the purification BPR and laboratory scale purification's showed that the levels of endotoxin were consistently within the acceptable range for human use when measured by gel clot and chromogenic LAL assays.

Production of low-endotoxin MSP1₄₂ began on April 12, 1999 at full scale (750g wet cell paste). Following the protein purification, the final product will be evaluated for endotoxin levels and induction of rabbit pyrogens. If endotoxin levels are in the acceptable range for this product, MSP1₄₂ will be tested for *in vivo* efficacy in nonhuman primate studies.

CONCLUSIONS

The presence of the native N-terminal sequences from MSP1₄₂ (MSP1₃₃) may have promoted proper folding and disulfide bond formation by initiating early productive folding pathways during protein translation. Expression of MSP1₄₂ (3D7) from the T7 promoter driven expression system avoided some problems previously found with bacterial expression of heterologous proteins, *i.e.* correct disulfide bond formation in the cytosolic reducing environment and partitioning of over-expressed recombinant heterologous proteins into insoluble inclusion bodies. The MSP1₄₂ protein was purified to near homogeneity following a three-column purification. Purified His₆-MSP1₄₂ protein was analyzed for identity and purity using SDS-PAGE and Coomassie Blue staining for total protein. Correct folding and disulfide bond formation of recombinant MSP1₄₂ was measured with a series of MSP1₁₉-specific conformation-dependent, reduction-sensitive mAbs and an MSP1₃₃-specific mAb on Western Blots (data not shown). These mAbs were developed against native parasite lysates and therefore they are relevant measures of native MSP1-conformation for specific epitopes on C-terminal MSP1. Future studies include evaluating MSP1₄₂ for its' ability to induce MSP1₄₂ specific antibodies and assessing *in vivo* efficacy in homologous challenge studies with *Aotus* monkeys.

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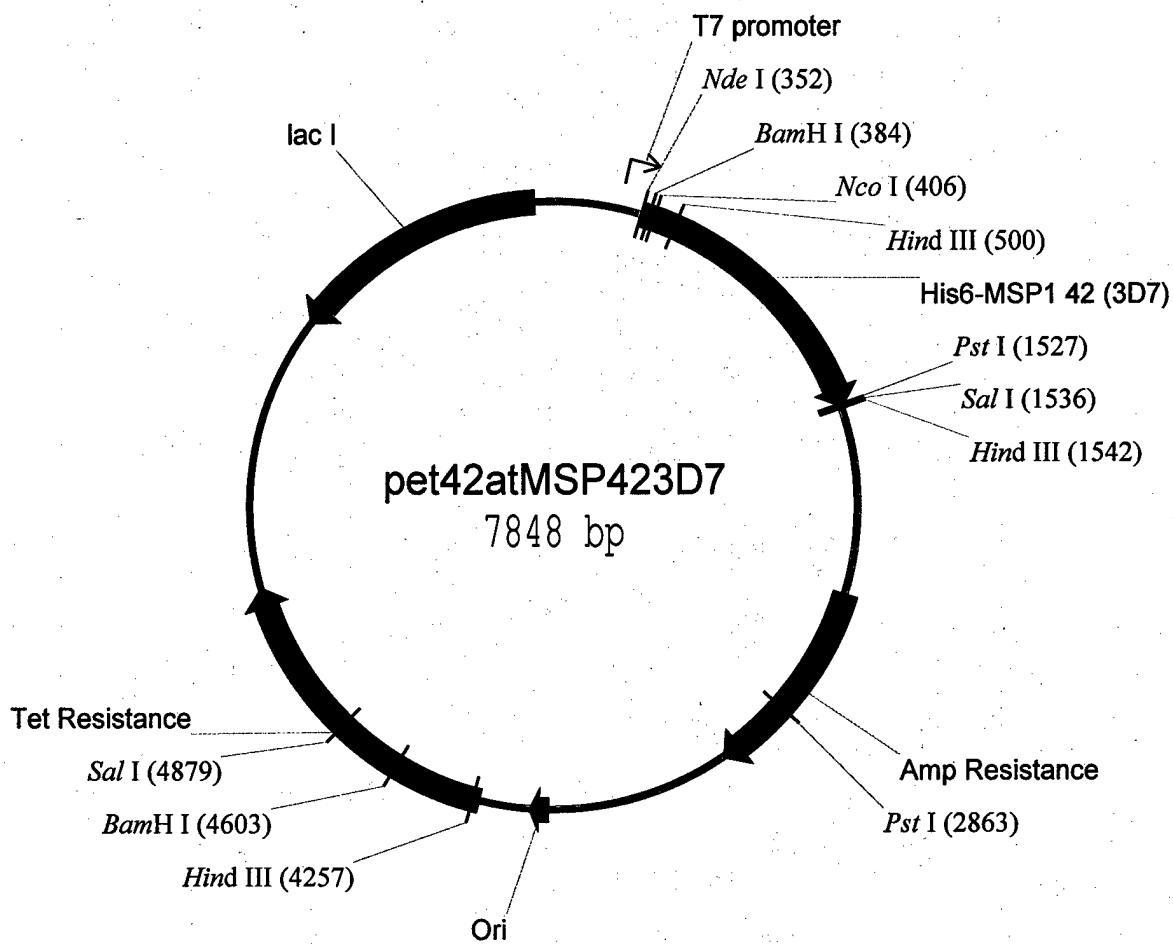
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2) Miami Nature Biotechnology Symposium, Miami, February 1-5, 1997.

Structural Analysis of Refolded-Recombinant *Plasmodium falciparum* MSP1 C-Terminal Fragment By Using Conformation-Specific Monoclonal Antibodies. Evelina Angov¹, Jana S. McBride², David C. Kaslow³, W.R. Ballou¹, Carter L. Diggs⁴, and Jeffrey A. Lyon¹. ¹Dept. Immunology, WRAIR, Washington, D.C., 20307; ²Division of Biological Sciences, Univ. Edinburgh, EH9 3JT, U.K.; ³NIAID, NIH, Bethesda, MD 20892; ⁴USAIL, Washington, D.C., 20523.



cGMP Purified, *E. coli* Expressed *P. falciparum* MSP1-42 (3D7)

